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Zishuo Cheng
Louisiana State University

Aimee Caillet
Louisiana State University

Binbin Ren
Louisiana State University

Huangen Ding
Louisiana State University

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Stimulation of *Escherichia coli* DNA damage inducible DNA helicase DinG by the single-stranded DNA binding protein SSB

Zishuo Cheng, Aimee Caillet, Binbin Ren, Huangen Ding *

Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803, USA

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ABSTRACT

***Escherichia coli* DNA damage inducible protein DinG is a superfamily II DNA helicase and is closely related to human DNA helicase XPD. Here, we report that *E. coli* single-stranded DNA binding protein (SSB) is able to form a stable protein complex with DinG and to stimulate the DinG DNA helicase activity. An SSB mutant that retains the single-stranded DNA binding activity but fails to form a protein complex with DinG becomes a potent inhibitor for the DinG DNA helicase, suggesting that *E. coli* wild-type SSB stimulates the DinG DNA helicase via specific protein–protein interaction.**

Structured summary of protein interactions:
SSB and **SSB** bind by molecular sieving (View interaction)
DinG and **SSB** bind by molecular sieving (View interaction)
DinG and **SSB** bind by cosedimentation in solution (View interaction)

1. Introduction

Escherichia coli gene *dinG* (DNA damage inducible gene G) is a member of the regulon induced by DNA damaging agents [1]. Purified *E. coli* DinG has an ATP-dependent helicase activity that unwinds double-stranded DNA [2], DNA–RNA duplex, D-loops, and R-loops [3]. Although the physiological function of DinG has not been fully understood, recent studies suggested that DinG may act to remove R-loops or together with other DNA helicases Rep and UvrD to promote replication across highly transcribed regions in *E. coli* genome [4]. Structurally, *E. coli* DinG belongs to superfamily II DNA helicases with 5′ to 3′ direction [2], and is closely related to yeast DNA helicase Rad3 [5] and human DNA helicases Xeroderma pigmentosum factor D (XPD) [6,7], FANCJ/BACH1 (BRCA1-associated C-terminal helicase) [8], CHLR1 (a DNA helicase involving in sister chromatid cohesion) [9], and RTEL1 (a regulator of telomere length) [10]. Furthermore, like yeast Rad3 [5] and human XPD [11–14], *E. coli* DinG contains a [4Fe–4S] cluster that is essential for the DNA helicase activity [15]. While the redox property and physiological role of the iron–sulfur cluster in XPD/Rad3 still remain elusive [16,17], we previously reported that the [4Fe–4S] cluster in *E. coli* DinG is stable and the DNA helicase activity remains fully active after the protein is exposed to 100-fold excess of hydrogen peroxide [15]. On the other hand, reduction of the [4Fe–4S] cluster in DinG reversibly switches off the DNA helicase activity, suggesting that the helicase activity could be regulated by intracellular redox potentials via the [4Fe–4S] cluster [15].

Exposure to DNA damaging agents would dramatically increase the number of single-stranded DNA (ssDNA) ends. In response, cells utilize the specialized ssDNA binding proteins (SSB) to protect ssDNA ends from further damage or re-annealing [18–20]. Importantly, recent studies further showed that SSB not only binds ssDNA but also interacts with a diverse group of DNA processing enzymes (see review [21]). Since both SSB and DinG are highly induced when *E. coli* cells are subject to DNA damaging agents [18], it would be of interest to explore the possible regulation of the DinG DNA helicase activity by SSB. In this study, we report that *E. coli* SSB is able to form a stable protein complex with DinG and to stimulate the DinG DNA helicase activity. A possible mechanism underlying the SSB-mediated stimulation of the DinG DNA helicase activity will be discussed.

2. Materials and methods

2.1. Protein preparation

A DNA fragment encoding the single-stranded DNA binding protein (SSB) was PCR-amplified from *E. coli* genomic DNA using

Abbreviations: DinG, *E. coli* protein encoded by the DNA damage inducible gene G; SSB, *E. coli* single-stranded DNA binding protein; XPD, human Xeroderma pigmentosum factor D

* Corresponding author. Fax: +1 225 578 2597.
E-mail address: hding@lsu.edu (H. Ding).

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two primers, SSB-1, 5′- GGAGACACGCATATGGCCAGCAGAG-3′, and SSB-2, 5′-ATTGTGCTAAGCACAAATCAGAACG-3′. The PCR product was digested with *Nde*I and *Blp*I, and ligated into an expression vector pET28b$^+$. The cloned DNA fragment was confirmed by DNA sequencing and introduced into an *E. coli* strain BL21. Recombinant SSB was overproduced in the *E. coli* cells grown in LB media under aerobic conditions. Cell extracts were treated with DNase (10 units/mL) to remove DNA before protein was purified as previously described in [15]. The N-terminal his-tag in SSB was removed by digestion with thrombin overnight and protein was re-purified using Mono-Q column. Purified SSB contains three extra amino acid residues (Gly-Ser-His) in N-terminus and an intact C-terminus which is responsible for specific interaction with multiple DNA processing proteins [21]. SSB mutant F177C (Phe-177 to Cys) was constructed using the Quikchange mutagenesis kit (Stratagene), and confirmed by DNA sequencing. SSB mutant protein was purified as described for wild-type SSB. Purified wild-type SSB and SSB mutant F177C showed the same ssDNA binding activity, as reported previously by others [22]. Recombinant *E. coli* DNA helicase DinG was purified as described in [15]. The purity of purified proteins was analyzed using SDS–polyacrylamide electrophoresis. The protein concentration of purified SSB and DinG was estimated from the absorption peak at 280 nm using an extinction coefficient of 27.9 and 78.7 $mM^{-1}cm^{-1}$, respectively. The bacteriophage single-stranded DNA binding protein gp32 [19] was purchased from New England BioLab.

2.2. Protein–protein interaction analyses

A gel filtration column (Superdex™ 200 (10/300GL)) attached to the ÄKTA FPLC system (GE Healthcare Life Sciences) was used for the protein complex analyses. The column was calibrated using the standard gel filtration protein markers (Sigma). For each run, protein sample (500 μL) was loaded onto the column and eluted with buffer containing NaCl (500 mM) and Tris (20 mM, pH 8.0) at a flow rate of 0.5 mL/min inside a 4 °C refrigerator. Eluted fractions (0.5 mL) were collected and aliquots were subject to the SDS–polyacrylamide electrophoresis.

The protein–protein interactions were also analyzed using the protein co-precipitation approaches following the procedure described in [23]. Unlike most proteins, *E. coli* SSB precipitates at 150 g/L ammonium sulfate. If a protein forms a complex with SSB, the protein will co-precipitate with SSB in the presence of 150 g/L ammonium sulfate in solution [23].

2.3. DNA helicase activity assay

The DNA helicase activity of *E. coli* DinG was analyzed following the procedure described by Voloshin et al. [2] with slight modifications [15]. Briefly, an oligonucleotide (5′-CCGTAACACTGAGT TTCGTCACCAGTACAAACTACAACGCCTGTAGCATTCCACA-3′) was labeled with ^{32}P-γ-ATP using polynucleotide kinase (New England BioLab). The ^{32}P-labeled oligonucleotide (0.2 μM) was annealed to M13mp18 ssDNA (0.1 μg/μL) (Fisher Scientific) in annealing buffer containing Tris (50 mM, pH 7.5), NaCl (50 mM) and $MgCl_2$ (10 mM). The DNA solution was heated at 85 °C for 5 min and cooled to room temperature over 3 h. The annealed DNA duplex was purified using a gel filtration spin-column Chromaspin 400 (Clontech co.) pre-equilibrated with annealing buffer. The annealed substrate (at a final concentration of 2 nM) was incubated with indicated concentrations of DinG protein in 20 μL the reaction solution containing Tris (50 mM, pH 7.5), NaCl (100 mM), $MgCl_2$ (5 mM), dithiothreitol (2 mM), glycerol (5%), and ATP (2 mM) at 30 °C for 10 min. For each experiment, two controls in which the substrate was either denatured by heating at 85 °C for 5 min or incubated at 30 °C for 10 min without any enzymes were included. The reactions were terminated by adding 4 μL stop solution (containing 6% SDS, 60 mM EDTA and 0.3% Bromophenol Blue). The reaction products were separated on 1% TAE agarose gel, transferred to nitrocellulose membranes, and exposed to X-ray films overnight for quantification of the reaction products.

3. Results and discussion

3.1. E. coli DinG forms a stable protein complex with single-stranded DNA binding protein SSB

To explore the possible interaction between the DNA-damage inducible proteins DinG and SSB [18], we purified both proteins from *E. coli* cells as described in Section 2. The SDS–PAGE gel analysis showed that both proteins were purified to a single-band (Fig. 1A). While purified DinG had an absorption peak at 403 nm of the [4Fe–4S] cluster [15], purified SSB only had the 280 nm protein absorption peak (Fig. 1B).

Fig. 2A shows the gel filtration profiles of purified SSB and DinG. While purified *E. coli* SSB formed a tetramer with an apparent molecular weight of ~134 kDa, as reported previously [19,24], purified *E. coli* DinG existed as a monomer with an apparent molecular weight of ~78 kDa. However, when a mix of DinG and SSB was loaded onto the gel filtration column, a new elution peak with an apparent molecular weight of ~200 kDa appeared. The SDS–PAGE analyses of eluted fractions showed that the new elution peak contained both DinG and SSB (Fig. 2A, bottom panel).

Because SSB and DinG are both the DNA binding proteins, any DNA contamination could contribute to formation of SSB/DinG complex. Using DNA indicator ethidium bromide, we were unable to detect any DNA in the protein samples. We also treated the protein samples with DNase before the gel filtration analyses, and found that the elution profiles were essentially identical when the protein samples were treated with or without DNase, further suggesting that formation of SSB/DinG complex does not depend on DNA.

E. coli SSB contains an N-terminal oligonucleotide/oligosaccharide binding domain serving as the ssDNA binding site and the C-terminal highly conserved end (Asp-Asp-Asp-Ile-Pro-Phe) involving in the protein–protein interaction with multiple DNA processing enzymes [21]. To examine whether the C-terminal end of SSB is involved in the protein–protein interaction with DinG, we constructed an *E. coli* SSB mutant in which the C-terminal end residue Phe-177 was replaced with Cys (F177C). Consistent with the previous report [22], we found that purified SSB mutant F177C formed a tetramer (Fig. 2B) and retained the same DNA binding activity as wild-type SSB (data not shown). However, when a mix of SSB mutant F177C and DinG was loaded onto the gel filtration column, a broad elution profile corresponding to the combination of the peaks of SSB mutant F177C and DinG was observed (Fig. 2B). The SDS–PAGE analyses of the eluted fractions confirmed that, unlike wild-type SSB, SSB mutant F177C failed to form a stable protein complex with DinG (Fig. 2B, bottom panel).

To further explore the protein–protein interaction between SSB and DinG, we adapted protein co-precipitation approaches following the procedures described in [23]. Unlike other proteins, SSB precipitates at a low concentration of ammonium sulfate in solution. Any protein that forms a stable protein complex with SSB would co-precipitate with SSB [23]. As shown in Fig. 3A, wild-type SSB co-precipitated a significant amount of DinG in the presence of 150 g/L ammonium sulfate. In contrast, SSB mutant F177C failed to co-precipitate any DinG under the same experimental conditions (Fig. 3B). Thus, wild-type SSB, but not SSB mutant Y177C, is able to form a stable protein complex with DinG via specific protein–protein interaction.

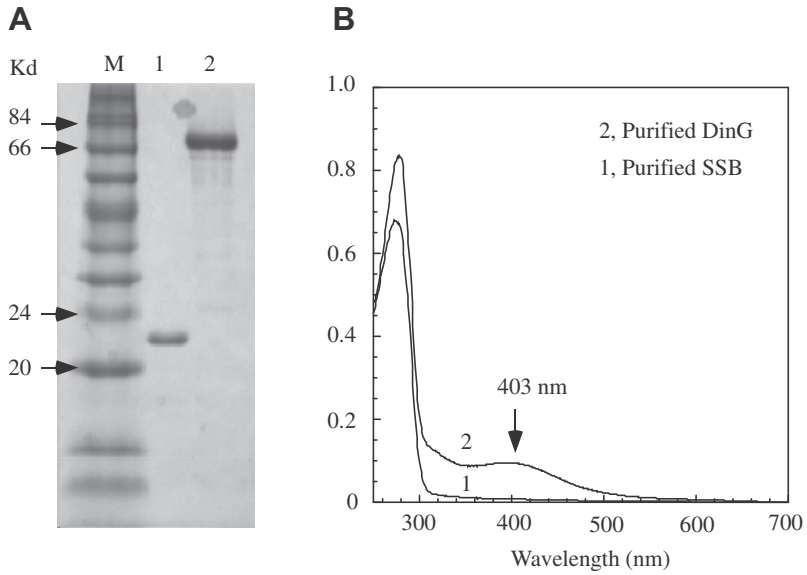


Fig. 1. Purification of *E. coli* DinG and SSB. (A) SDS–PAGE gel of purified *E. coli* DinG and SSB. Lane M, molecular weight markers; lane 1, purified SSB; lane 2, purified DinG. (B) UV–vis absorption spectrum of purified *E. coli* SSB (spectrum 1) and DinG (spectrum 2). The proteins were dissolved in buffer containing NaCl (500 mM) and Tris (20 mM, pH 8.0). The contraction of SSB and DinG shown in (B) was 22 and 10 μM, respectively.

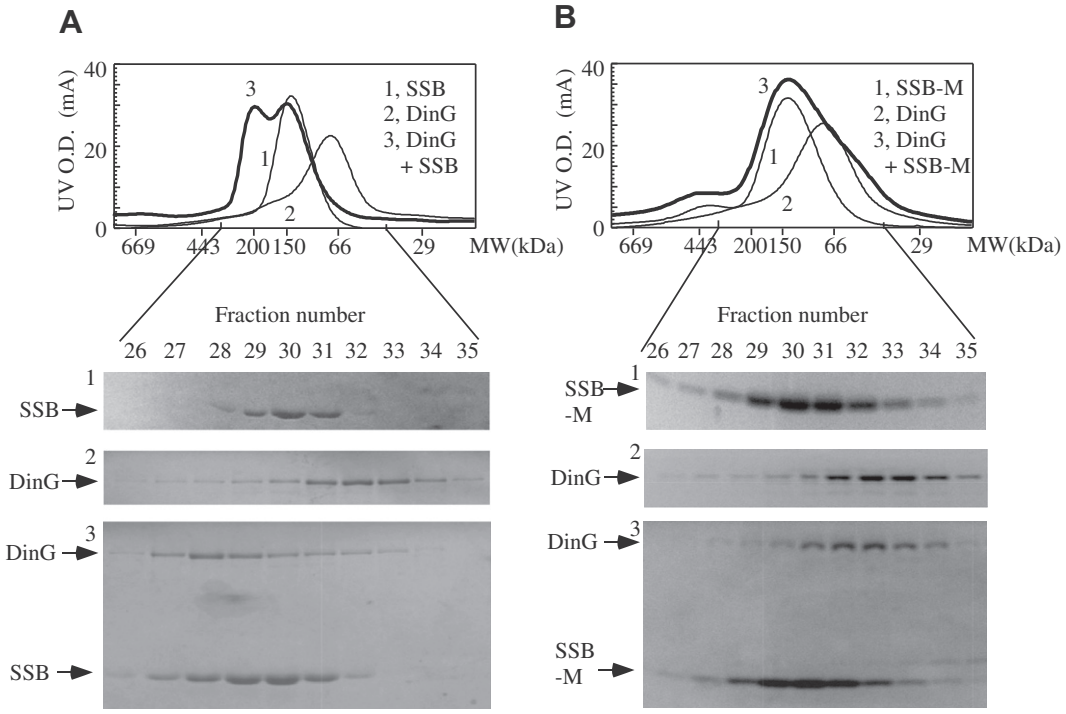


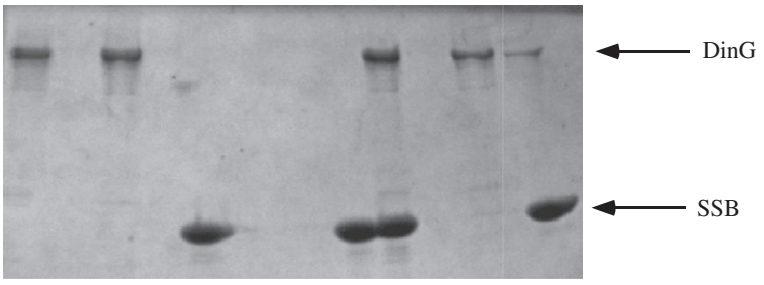
Fig. 2. Gel filtration analyses of the SSB/DinG protein complex. (A) Gel filtration profiles of SSB, DinG and a mix of SSB and DinG. Top panel, gel filtration profiles of SSB (80 μM) (trace 1), DinG (20 μM) (trace 2), and a mix of SSB (80 μM) and DinG (20 μM) (trace 3). The proteins were dissolved in buffer containing NaCl (500 mM) and Tris (20 mM, pH 8.0) and eluted from the gel filtration column using the same buffer. The molecular weights of the standard gel filtration protein markers were labeled on *x*-axis. Bottom panel, SDS gel photos of the fractions (26–35) eluted from the gel filtration column. The protein bands were indicated on the left side. (B) Gel filtration profiles of SSB mutant F177C, DinG and a mix of SSB mutant F177C and DinG. Top panel, gel filtration profiles of SSB mutant F177C (SSB-M) (80 μM) (trace 1), DinG (20 μM) (trace 2), and a mix of SSB-M (80 μM) and DinG (20 μM) (trace 3). The molecular weights of the standard gel filtration protein markers were labeled on *X*-axis. Bottom panel, SDS gel photos of the fractions (26–35) eluted from the gel filtration column. The protein bands were indicated on the left side. Data are representative of three independent experiments.

3.2. E. coli SSB enhances the DinG DNA helicase activity

Formation of SSB/DinG complex led to an idea that SSB may modulate the DinG DNA helicase activity via protein–protein interaction. Using the previously established DNA helicase activity assay [2], we explored the effect of SSB on the DinG DNA helicase activity. Fig. 4A shows that addition of SSB indeed stimulated the DinG DNA helicase activity by at least two folds. We also analyzed the DinG DNA helicase activity in the presence of a fixed concentration of DinG and increasing concentrations of SSB, and found that as the SSB concentration was gradually increased, the DinG DNA helicase activity was progressively increased (Fig. 4B). A 5–10-fold excess of SSB required for stimulating the DinG DNA helicase activity (Fig. 4B) could be due to the substrate

A

+DinG	+	+	+	+					+	+	+	+
+SSB					+	+	+	+	+	+	+	+
+$(NH_4)_2SO_4$			+	+			+	+			+	+
S/P	S	P	S	P	S	P	S	P	S	P	S	P
Lane	1	2	3	4	5	6	7	8	9	10	11	12



B

+DinG	+	+	+	+					+	+	+	+
+SSB-M					+	+	+	+	+	+	+	+
+$(NH_4)_2SO_4$			+	+			+	+			+	+
S/P	S	P	S	P	S	P	S	P	S	P	S	P
Lane	1	2	3	4	5	6	7	8	9	10	11	12

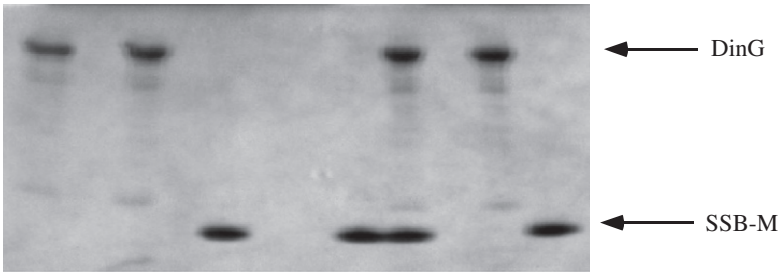


Fig. 3. Protein co-precipitation analyses of SSB and DinG. *E. coli* DinG (20 μM), SSB (panel A) or SSB mutant F177C (pane B) (80 μM) in buffer containing Tris (10 mM, pH 7.2) NaCl (150 mM), and glycerol (10% (v/v)) was incubated with ammonium sulfate (150 g/L) in various solutions as indicated by + symbols. After incubation, samples were centrifuged. Pellet (P) and supernatant (S) fractions were loaded on the SDS–polyacrylamide gel. The results are representative of three independent experiments.

ssDNA M13 plasmid which may titrate out SSB in the reaction solution. Nevertheless, the results clearly suggest that *E. coli* SSB is able to stimulate the DinG DNA helicase activity under the experimental conditions.

3.3. SSB mutant F177C is a potent inhibitor for the DinG DNA helicase

As a single-stranded DNA binding protein, SSB may regulate the DinG DNA helicase activity by binding to ssDNA, a substrate/product of the DNA helicase. If a protein that binds ssDNA could stimulate the DinG DNA helicase activity, we expect that SSB mutant F177C which retains the same ssDNA binding activity as wild-type SSB should also stimulate the DinG DNA helicase activity.

To our surprise, unlike wild-type SSB, SSB mutant F177C effectively inhibited the DinG DNA helicase activity (Fig. 5A). To further explore whether other ssDNA binding proteins could inhibit the DinG DNA helicase activity, we used bacteriophage protein gp32, a structurally unrelated ssDNA binding protein [19] and found that gp32 had an even stronger inhibitory effect on the DinG DNA helicase activity (Fig. 5B). Thus, the specific protein–protein

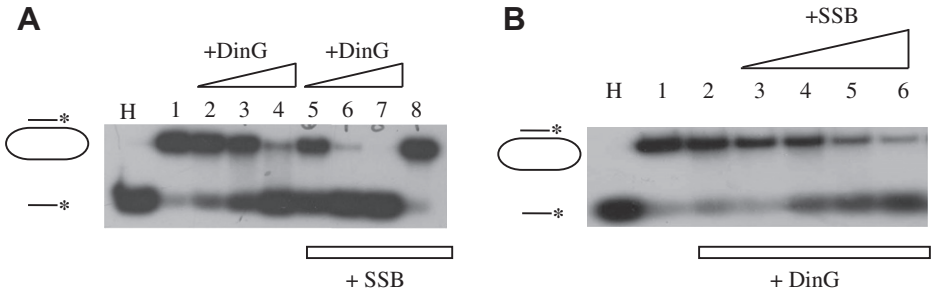


Fig. 4. *E. coli* SSB stimulates the DinG DNA helicase activity. (A) Purified DinG was incubated with the ^{32}P-radioactive-labeled substrate and ATP with or without SSB at 30 °C for 15 min. Lane H, the sample was heated at 85 °C for 5 min. Lane 1, no DinG. Lanes 2–4, with 25, 50 and 100 nM DinG. Lanes 5–7, with 1 μM SSB and 25, 50 and 100 nM DinG). Lane 8, no DinG and 1 μM SSB only. (B) Purified DinG was incubated with the ^{32}P-radioactive-labeled substrate (2 nM), ATP (2 mM) and SSB at 30 °C for 15 min. Lane H, the sample was heated at 85 °C for 5 min. Lane 1, no DinG or SSB. Lanes 2–6, with 50 nM DinG and 0, 50, 100, 250 and 500 nM SSB, respectively. Similar results were obtained from three independent experiments.

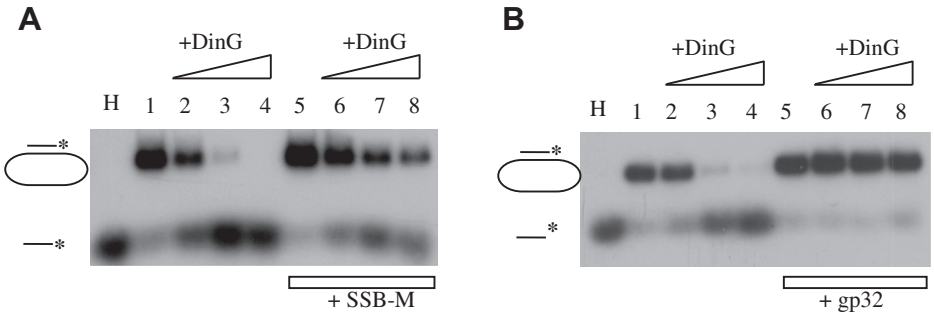


Fig. 5. SSB mutant F177C inhibits the DinG DNA helicase activity. (A) Purified DinG was incubated with the ^{32}P-radioactive-labeled substrate (2 nM) and ATP (2 mM) with or without SSB mutant F177C at 30 °C for 15 min. Lane H, the sample was heated at 85 °C for 5 min. Lane 1, no DinG. Lanes 2–4, with 50, 100 and 200 nM DinG. Lanes 5–8, with 1 μM SSB mutant F177C and 0, 50, 100, and 200 nM DinG. (B) Purified DinG was incubated with the ^{32}P-radioactive-labeled substrate (2 nM) and ATP (2 mM) with or without bacteriophage protein gp32 at 30 °C for 15 min. Lane H, the sample was heated at 85 °C for 5 min. Lane 1, no DinG. Lanes 2–4, with 50, 100 and 200 nM DinG. Lanes 5–8, with 500 nM gp32 and 0, 50, 100, and 200 nM DinG. Data are representative of three independent experiments.

interaction between wild-type SSB and DinG is likely responsible for stimulation of the DinG DNA helicase activity by SSB. On the other hand, the ssDNA binding activity of SSB appears to inhibit the DinG DNA helicase activity.

The observation that wild-type SSB and SSB mutant F177C have an opposite effect on the DinG DNA helicase activity demonstrates the crucial role of the C-terminal end F-177 in SSB. It has been reported that mutation of F177C in SSB severely impairs the *E. coli* cell's viability [22], and F177 may directly interact with multiple DNA processing enzymes [21]. Here we show that SSB mutant F177C, which retains the ssDNA binding activity as wild-type SSB [22], fails to form a stable SSB/DinG complex. We envision that formation of SSB/DinG complex may subtly alter the structure of both proteins: for DinG, binding of SSB may lead to an enhanced DNA helicase activity; for SSB, binding of DinG may weaken the ssDNA binding activity. As a consequence, specific protein–protein interaction between SSB and DinG stimulates the DinG DNA helicase activity. In contrast, SSB mutant F177C does not form a stable protein complex with DinG, thus fails to stimulate the DinG DNA helicase activity. Instead, the ssDNA binding activity of SSB mutant F177C effectively blocks the access of DinG to substrate ssDNA and inhibits the DinG DNA helicase activity. In line with this idea, we found that while wild-type SSB can enhance the endogenous ATPase activity of DinG, SSB mutant F177C effectively inhibits the ATPase activity of DinG (unpublished data). Nevertheless, additional experiments are required to illustrate molecular details of the SSB-mediated activation of the DinG DNA helicase activity.

The known proteins that interact with *E. coli* SSB include the primase for DNA replication DnaG [25], exonuclease I [26], the DNA helicase RecQ [23,24], uracil DNA glycosylase [27], the χ subunit of DNA polymerase III [28], DNA polymerase V [29], topoisomerase III [30], the replication re-start protein DNA helicase PriA [31], DNA helicase RecG [32], recombination mediator RecO [33,34], and the maintenance of genome stability protein A [35]. In a number of the SSB-binding proteins, a hydrophobic pocket and basic residues have been identified for accommodation of the C-terminal end Phe-177 and Asp residues of SSB [21,24,33,36]. In Gram-positive *Bacillus subtilis*, SSB has also been shown to recruit DNA helicases PriA and RecG and recombination mediator RecO, and to re-start the arrested chromosomal replication forks [37]. In archaea, the single-stranded DNA binding protein RPA (Replication Protein A) has been shown to interact with DNA helicase XPD [38,39] and RNA polymerase [40]. In eukaryotes, RPA interacts with DNA polymerase α [41] and DNA helicase FANCJ/BACH1 [42,43], and is likely responsible for coordinating repair of double-stranded DNA breaks [44]. In this context, we propose that *E. coli* DinG is a new member of the DNA processing protein family that can be regulated by SSB. When cells are subject to DNA damaging agents, DinG together with other DNA repair proteins including SSB are highly induced [1,18], and SSB in turn stimulates the activity of the DinG DNA helicase and other DNA repair enzymes to promote efficient repair of DNA damage.

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